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Microbial Sulfate Reduction and Its Potential Utility as an Acid Mine Water Pollution Abatement Procedure

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The presence of high concentrations of sulfate, iron, and hydrogen (acid) ions in drainage from coal mines and other areas containing waste pyritic materials is a serious water pollution problem. Sulfate can be removed from solution by microbial reduction to sulfide and subsequent precipitation as FeS. A mixed culture of microorganisms degraded wood dust cellulose, and the degradation products served as carbon and energy sources for sulfate-reducing bacteria. Metabolism of carbon compounds resulted in a net *p*H increase in the system. Oxidation-reduction potential (Eh) and temperature and carbon supplements were studied in an effort to accelerate the sulfate reduction process, with the ultimate objective of utilizing the process as a pollution abatement procedure.

High concentrations of sulfate, iron, and hydrogen ions are characteristic of acid mine drainage, and it has been shown that sulfate in association with acid exerts a deleterious influence on heterotrophic bacteria present in nonacid receiving streams (3).

In an earlier investigation, Moulton et al. showed that mixed cultures of sulfate-reducing bacteria increased the *p*H of a lactic acid-mineral salts medium, containing sulfuric acid, from 5 to 8.9 in 8 days at room temperature (4). From these data, it was thought that wood dust (sawdust) or sewage might provide nutrients and a sufficiently low oxidation-reduction (O/R) potential for the natural establishment of sulfate-reducing bacteria, and that these bacteria could be used to dispose of sulfur-containing waste materials in water of the type that is discharged from abandoned coal mines.

The activity of anaerobic sulfate-reducing bacteria has been studied under natural conditions in acid mine drainage as well as under controlled laboratory conditions. The carbon and energy supply for sulfate reduction by mixed cultures of anaerobic bacteria was wood dust, and the ecosystem involved in cellulose decomposition as well as in sulfate reduction has been discussed (7). Under controlled temperature and wood dust concentration, sulfate could be removed from acid mine water with a concomitant increase in *p*H.

This report considers sulfate reduction in mixed culture systems as a potential treatment process or acid drainage abatement procedure. Sulfate is supplied by acid mine drainage or $MgSO_4$ salt solutions, and carbon is supplied as wood dust. Particular emphasis is placed on the physical and chemical parameters which appear to be pertinent to the activity of sulfate-reducing bacteria in acid mine water.

MATERIALS AND METHODS

Chemical determinations. Total dissolved iron was measured colorimetrically by the phenanthroline method according to the procedure described for a Hach Field Kit (Hach Chemical Co., Des Moines, Iowa).

Sulfate was determined turbidimetrically as $BaSO_4$ precipitate, as described by the Hach procedure. O/R potentials (or Eh) and *p*H were determined with a Corning expanded scale *p*H meter (Corning Glass Co., Corning, N.Y.).

Media and growth conditions. Sulfate-reducing bacteria were enumerated with a standard three-tube most probable number (MPN) method (1), by the tube culture technique described by Postgate (5) with *Desulfovibrio desulfuricans* medium no. 3. Positive tubes were black after incubation at 25 ± 2 C for 6 to 21 days.

Enrichment cultures of sulfate-reducing bacteria which contained mixed populations were partially purified by isolation on plates of *D. desulfuricans* medium no. 3 (5) which had been overlaid with 1.5% water-agar. Black colonies which appeared after incubation at 37 C in 1 atm of 95% N_2-5% CO_2 were inoculated into 8-oz (0.236-liter) prescription bottles containing medium "C" of Butlin et al. (2). The cultures were incubated at 37 C and then held at ambient temperature for future use.

Wood dust cultures. Wood dust-acid mine water

cultures were prepared by placing 1 liter of water (*p*H 3.6), obtained from an abandoned drift mine (Ohio no. 47), and 400 g of wood dust into a 2-liter Erlenmeyer flask. Wood dust was obtained from the surface (nondegraded) of a large wood dust pile and also from a depth of 3 ft (0.914 m; partially degraded wood dust) into the pile. The pile was adjacent to a mill which cut hardwoods, primarily oak. Cultures containing each wood dust sample were incubated at 25 ± 2 C, 37 C, and 50 C. A duplicate set of cultures, in which 890 μg of sulfate (as $MgSO_4 \cdot 7H_2O$) per ml of water replaced acid mine water, was incubated as above. The wood dust tended to settle during incubation, and the overlying solutions were periodically examined for sulfate, iron, and hydrogen ion concentrations.

Interrelationships among chemical parameters. Flask cultures containing partially degraded wood dust and acid mine water were prepared as described above. Sodium lactate was added to the flask to give a 0.1% concentration (w/v), and the mixed cultures were seeded with 25 ml of a 7-day culture of mixed sulfate-reducing bacteria to increase the population of sulfate-reducing bacteria. The cultures were incubated at 37 C. O/R, *p*H, and dissolved iron and sulfate concentrations were determined at suitable time intervals in a 25-ml sample which was removed aseptically. Care was taken not to agitate the sample before O/R determination.

Addition of exogenous substrates. Flask cultures containing 400 g of partially degraded wood dust and 1 liter of $MgSO_4$ solution (800 μg of sulfate per ml of water) were prepared as described above. Individual culture flasks were supplemented with various carbon sources as follows: 0.01, 0.1, and 1.0% glucose; 1.0% xylose; 0.1 and 1.0% sodium butyrate; 0.1% propionic acid; 0.1% acetic acid; 0.1% sodium formate, 1.0% succinic acid; and 0.1% acetone. One flask which did not contain an added carbon source was held as a control. The cultures were incubated at 37 C, and portions were assayed for sulfate at suitable time intervals.

RESULTS

Effect of temperature and wood dust condition on sulfate reduction. Table 1 shows the effect of wood dust condition [fresh versus partially degraded from a 3-ft (0.914 m) depth in the wood dust pile] on sulfate removal from acidic mine water. Sulfate removal implies sulfate reduction to sulfide and either precipitation of sulfide as black FeS or loss from the system as H_2S gas. Table 1 also shows the effect of incubation temperature on sulfate concentration in wood dust cultures containing acid mine water (initial *p*H 3.6 to 3.8). More sulfate was reduced at 37 C than at either ambient temperature or 50 C over a 14-day period. Cultures at 50 C reduced sulfate more rapidly and to a greater total extent than did ambient cultures, regardless of the wood dust condition. Although maximal total sulfate reduction occurred at 37 C in the presence of partially degraded wood dust, the maximal rate of sulfate reduction occurred at 37 C when fresh wood dust was present. This could result from differences in the substrate concentration which was initially available to the sulfate reducers in fresh wood dust as compared to partially degraded wood dust. That is, a longer lag period occurred when fresh wood dust was the substrate. The partially decomposed wood dust appeared to allow a more consistent rate of sulfate reduction over a longer time period.

The influence of substituting $MgSO_4$ (initial *p*H 4.2 to 5.8) for acid mine water is shown in Table 2, when the values are compared to those in Table 1. When partially degraded wood dust was the substrate for the organisms, there was a slight increase in sulfate reduction values in $MgSO_4$ at ambient temperature and at 50 C and a somewhat greater increase at 37 C. There was a marked increase in both the rate and total amount of sulfate reduced when $MgSO_4$ was substituted for mine water at 37 and at 50 C when fresh wood dust was the carbon substrate. This may be attributable to the initial *p*H difference. No sulfate reduction was observed at ambient temperature in fresh wood dust. This may be due to a lack of nutrients, normally found in acid mine water, which are required by the mesophilic wood dust decomposers. The iron concentration in the $MgSO_4$ cultures was never in excess of 2.5 μg/ml; iron was present as a constituent of the wood dust but was not added intentionally.

Chemical and physical parameters during growth in mixed culture. Figure 1 shows changes in four parameters during growth of the mixed culture system in wood dust-acid mine water which had been enriched with 0.1% sodium lactate. The *p*H increased from 3.6 to 7.0 during a 10-day period. Eh (O/R) continually decreased, but had a change in rate of decrease at about the 4th day. The solution potential became negative between the 4th and the 5th day.

At approximately the same time, the slope of the *p*H curve increased and an abrupt alteration in the rate of sulfate removal became evident. The concentration of dissolved iron increased rapidly for the first 6 days. This can be attributed to increased solubility of ferrous iron as the potential dropped. After 6 days, the Eh approached 200 mv and the sulfate removal proceeded at a maximum rate. Iron decrease was concomitant with sulfate reduction during this time period because of precipitation of black FeS.

Exogenous substrate supplements. The effect of

TABLE 1. *Comparison of the effects of wood dust quality (i.e., the extent of wood dust degradation by microorganisms) and incubation temperature on the reduction of sulfate and removal of H^+ from acid mine water in untreated wood dust cultures containing 400 g of wood dust and 2 liters of water*[a]

Determination	Nondegraded wood dust			Partially degraded wood dust		
	Ambient temp (22 ± 2 C)	37 C	50 C	Ambient temp (22 ± 2 C)	37 C	50 C
Amt of sulfate (μg) removed per ml of water after 14 days	30	480	65	290	590	460
Maximal rate of sulfate removed (μg) per ml of water per day	10.0	71.7	30.0	19.4	53.2	38.8
*p*H range, 0 to 14 days	3.6 to 4.2	3.7 to 5.8	3.8 to 4.2	3.5 to 4.3	3.5 to 6.4	3.7 to 5.8

[a] Each culture contained acid mine water with an initial sulfate concentration of 890 μg of sulfate per ml of water.

TABLE 2. *Comparison of the effect of wood dust quality and incubation temperature on the reduction of sulfate and removal of H^+ from a $MgSO_4$ solution containing 400 g of wood dust and 2 liters of water*[a]

Determination	Nondegraded wood dust			Partially degraded wood dust		
	Ambient temp (22 ± 2 C)	37 C	50 C	Ambient temp (22 ± 2 C)	37 C	50 C
Amt of sulfate (μg) removed per ml of water in 14 days	0	715	140	290	700	480
Maximal rate of sulfate removed (μg) per ml of water per day	0	80.0	56.0	27.5	58.3	62.5
*p*H range, 0 to 14 days	4.2 to 4.7	4.2 to 5.0	4.5 to 4.8	5.8 to 6.4	5.8 to 7.3	5.2 to 7.3

[a] The initial sulfate concentration was 890 μg per ml of water.

carbon substrates added to wood dust cultures on sulfate removal is presented in Table 3. Several substrates increased sulfate reduction, whereas others retarded or inhibited reduction. In a concentration[1] of 0.1%, glucose stimulated sulfate removal, whereas a 1.0% concentration retarded sulfate removal when compared to a control. Incubation of the glucose culture for an additional 12 days did not permit sulfate reduction. Sodium formate allowed sulfate reduction, but never at the rate of the control culture. Although 18 days of incubation of the succinate culture was not sufficient to cause sulfate reduction, prolonged incubation resulted in a rate of sulfate reduction greater than that in the control culture.

MPN determinations of sulfate-reducing bacteria made after a 12-day incubation period correlated with sulfate reduction (Table 4).

The inhibition or retardation of sulfate reduction appeared to be due to either an increased lag period or a different rate of removal. Figures 2 and 3 present curves showing sulfate removal versus time in wood dust-acid mine water cultures which were supplemented with various carbon sources. Figure 2 shows that lactic acid and succinic acid increased the rate (slope) of sulfate removal as compared to control curves. However, a long lag period occurred in the presence of succinic acid. This may be due to a higher O/R potential rather than to a direct inhibitory effect. Figure 3 shows results of an experiment which was essentially the same as that shown in Fig. 2, except that different carbon sources were added. The similar slopes observed with propionate and the control indicate that propionate slightly stimulated sulfate reduction by decreasing the lag period. Butyrate stimulated sulfate reduction by both decreasing the lag period and

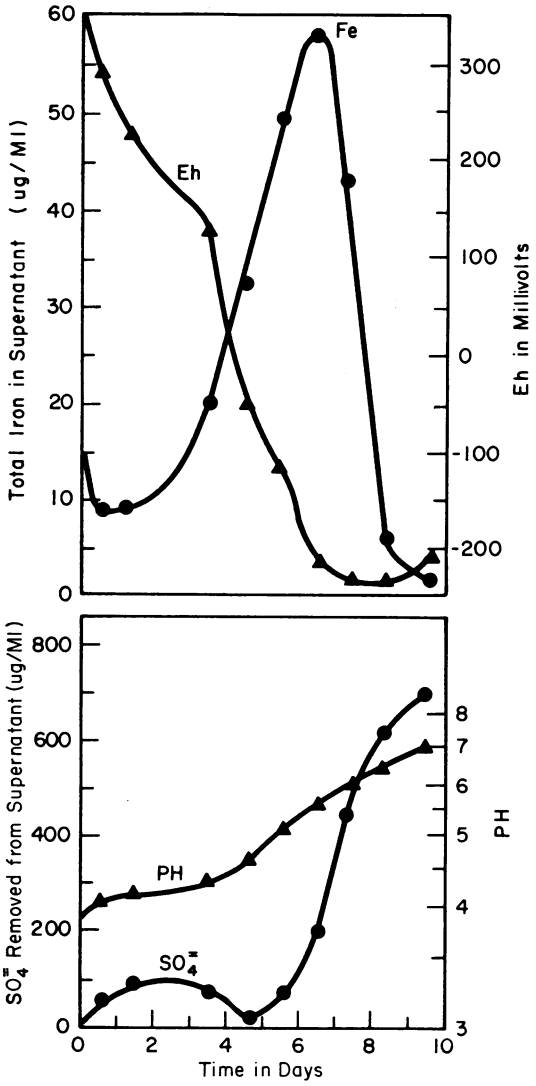

FIG. 1. *Change in Eh, pH, and dissolved iron and sulfate concentration versus time, during growth of a mixed culture at 37 C in acid mine water containing wood dust and 0.1% sodium lactate.*

increasing the rate of reduction (slope). The data presented in Fig. 2 and 3 represent two separate experiments, and the differences in the control curves shown in Fig. 2 and 3 probably reflect slight differences in the composition of the wood dust added to the flasks.

DISCUSSION

Two primary difficulties must be overcome to accomplish sulfate reduction in acidic water. Dissimilatory sulfate-reducing bacteria require an O/R potential of −150 to −200 mv (6); therefore, the water must be made anaerobic. Secondly, a source of organic nutrients to supply energy and carbon for the heterotrophic anaerobes is required. Although we previously reported (8) that the numbers of anaerobic microorganisms in acid mine water are low, the addition of organic materials is favorable to the establishment of an anaerobic, heterotrophic microflora in acidic water (7). This process is illustrated graphically in Fig. 1. Wood-dust degradation is necessary for the establishment of an anaerobic microflora and, in particular, for sulfate-reducing bacteria. The rate of wood-dust

TABLE 3. *Effect of the addition of exogenous substrates on sulfate reduction in wood dust cultures*[a]

Substrate	Concn (%)	Per cent increase of sulfate reduction[b]
Glucose	1.0	0[c]
Glucose	0.1	83
Glucose	0.01	0
Xylose	1.0	0[c]
Sodium butyrate	0.1	137
Propionic acid	0.1	34
Acetic acid	0.1	51
Sodium formate	0.1	0[c]
Succinic acid	1.0	0[c]
Acetone	0.1	0

[a] Each culture contained 400 g of partially degraded wood dust and 2 liters of an $MgSO_4$ solution adjusted to 800 μg of sulfate per ml of water. Substrates were added to give the final per cent concentrations (w/v) shown and were incubated at 37 C. The per cent increase in sulfate reduction represents the difference between the amount of sulfate reduced in the culture containing an exogenous substrate and a control culture which contained 800 μg of $MgSO_4$ per ml of solution plus wood dust. Values are expressed as per cent increase in sulfate reduction over the control. Inhibition indicates that less sulfate was reduced in the culture containing added substrate than in the control culture.

[b] In an 800 μg/ml $MgSO_4$ solution after 14 days of incubation at 37 C.

[c] Inhibition.

TABLE 4. *Enumeration of sulfate-reducing bacteria in culture after 12 days at 37 C*

Substrate added in addition to wood dust	No. of bacteria (MPN/100 ml)
1.0% Glucose	2.3×10^4
1.0% Succinic acid	9.1×10^3
1.0% Lactic acid	4.6×10^6
None	4.6×10^5

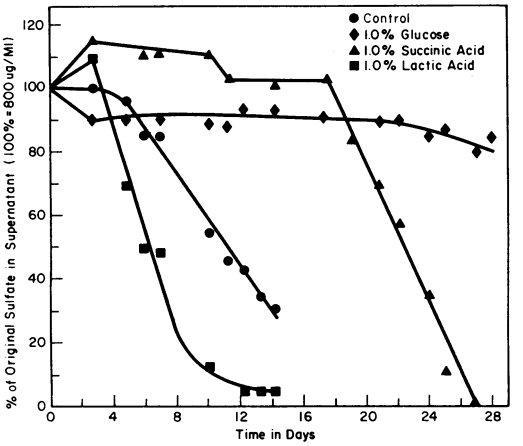

FIG. 2. *Rate of dissolved sulfate loss versus time, during growth of a mixed culture at 37 C in acid mine water containing wood dust (control) and in the control solution plus glucose, succinic acid, or lactic acid.*

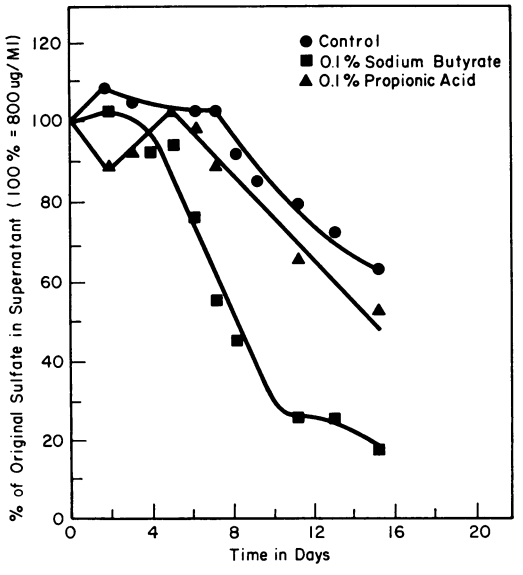

FIG. 3. *Rate of dissolved sulfate loss versus time, during growth of a mixed culture containing acid mine water and wood dust (control) and in the control solution plus sodium butyrate or propionic acid.*

degradation therefore appears to control both the initiation and the rate of sulfate reduction. Several physiological types of microorganisms (both aerobes and anaerobes) undoubtedly participate in the wood dust degradation process according to proposed degradative schemes presented previously (7). It has also been reported that temperature is a primary factor affecting wood dust breakdown and that the temperatures most favorable for this process may not yield maximal rates of sulfate reduction. This is a result of differing temperature optima among the responsible physiological groups of bacteria; e.g., the sulfate-reducing bacteria in our system grew best at 37 C, wood-dust degradation occurred most rapidly at 50 C.

The energy requirements of the sulfate-reducing bacteria must also be considered. Both butyrate and lactate served as energy sources for our isolates in artificial culture media (*unpublished data*). Other sulfate-reducing bacteria have been shown to be capable of oxidizing straight-chain alcohols, pyruvate, and choline (6), and acetate is commonly formed as the end product of the fermentation. The data (Table 3) suggest that acetate might be recycled in the wood dust system. This may be accomplished by other anaerobes, e.g., *Clostridium* species. The same explanation may fit the observed enhancement of sulfate reduction by propionic acid.

The system may be upset by the excess addition of substrates which enhance sulfate reduction in low concentrations. This is shown by the concentration phenomenon observed for glucose (Table 3). This effect may either be a result of an interruption in the cellulose breakdown process, causing a shift in microflora which do not produce substrate for sulfate-reducing bacteria, or a result of stimulation of a microflora which may effectively compete with sulfate reducers for nutrients which were produced from glucose. Note that viable sulfate-reducing bacteria were present in cultures in which measurable sulfate reduction did not occur (Table 4) and that these anaerobes do not attack glucose (6).

Wood dust quality also influences the rate of sulfate reduction. The maximal rates of sulfate reduction occurred in nondegraded wood dust at 37 C (Tables 1 and 2). Because a longer lag period was observed in fresh wood dust cultures than in partially degraded wood dust, the apparent equality of total sulfate removal in all cultures at 37 C is misleading. In practical use, the lag period is not an important consideration, since the conditions during this period are more similar to a continuous culture than to a batch culture.

The data suggest (Table 2) that iron is not required in dissimilatory quantities for sulfate reduction in the mixed culture system, although sulfate-reducing bacteria do require iron (6). Constant influx of acid mine water into the system would be expected to furnish sufficient iron and sulfate for these bacteria.

Although much of the iron and sulfate could be removed, further water treatment would be

necessary for use as a treatment procedure. Precipitated iron sulfides in a mine water system would have to be removed in order to prevent natural reoxidation of iron and sulfide (as well as oxidation resulting from *Thiobacillus* species which are indigenous to acid mine drainage), if practical use is to be made of the process. One possible inducement may be with recovery of sulfur as FeS or as elemental sulfur if an additional microbiological conversion step was introduced into the system. Although no data concerning larger systems are presented in this paper, it has been possible to remove sulfate in a continuous system by using 5-gal (18.9 liters) holding tanks for the wood dust culture and by flowing acid mine water through the culture. There appears to be no reason why other waste carbon materials could not substitute for wood dust, e.g., sewage, waste paper, algae, aquatic weeds, or any waste vegetable material. It may be possible to combine efforts designed to alleviate certain types of solid waste removal or other types of water pollution abatement with the acid mine drainage abatement problem. It should also be possible either to utilize abandoned coal mines in certain cases as fermentation vessels or to design anaerobic digesters to carry out the sulfate reduction process.

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